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Heterotetrameric Sarcosine Oxidase: Structure of a Diflavin Metalloenzyme at 1.85 Å Resolution

Zhi-wei Chen[1], Alshaimaa Hassan-Abdulah[2], Gouhua Zhao[2] Marilyn Schuman Jorns[2]* and F. Scott Mathews[1]*

[1]*Washington University School of Medicine, St. Louis, MO 63110, USA*

[2]*Drexel University College of Medicine, Philadelphia, PA 19102, USA*

**Corresponding authors*

The crystal structure of heterotetrameric sarcosine oxidase (TSOX) from *Pseudomonas maltophilia* has been determined at 1.85 Å resolution. TSOX contains three coenzymes (FAD, FMN and NAD^+), four different subunits (α, 103 kDa; β, 44 kDa; γ, 21 kDa; δ, 11 kDa) and catalyzes the oxidation of sarcosine (*N*-methylglycine) to yield hydrogen peroxide, glycine and formaldehyde. In the presence of tetrahydrofolate, the oxidation of sarcosine is coupled to the formation of 5,10-methylenetetrahydrofolate. The NAD^+ and putative folate binding sites are located in the α-subunit. The FAD binding site is in the β-subunit. FMN is bound at the interface of the α and β-subunits. The FAD and FMN rings are separated by a short segment of the β-subunit with the closest atoms located 7.4 Å apart. Sulfite, an inhibitor of oxygen reduction, is bound at the FMN site. 2-Furoate, a competitive inhibitor with respect to sarcosine, is bound at the FAD site. The sarcosine dehydrogenase and 5,10-methylenetetrahydrofolate synthase sites are 35 Å apart but connected by a large internal cavity (~10,000 $Å^3$). An unexpected zinc ion, coordinated by three cysteine and one histidine side-chains, is bound to the δ-subunit. The N-terminal half of the α subunit of TSOX (αA) is closely similar to the FAD-binding domain of glutathione reductase but with NAD^+ replacing FAD. The C-terminal half of the α subunit of TSOX (αB) is similar to the C-terminal half of dimethylglycine oxidase and the T-protein of the glycine cleavage system, proteins that bind tetrahydrofolate. The β-subunit of TSOX is very similar to monomeric sarcosine oxidase. The γ-subunit is similar to the C-terminal sub-domain of α-TSOX. The δ-subunit shows little similarity with any PDB entry. The αA domain/β-subunit sub-structure of TSOX closely resembles the αβ dimer of L-proline dehydrogenase, a heteroctameric protein $(\alpha\beta)_4$ that shows highest overall similarity to TSOX.

Keywords: crystal structure; sarcosine oxidase; flavin mononucleotide; flavin adenine dinucleotide; tetrahydrofolate

Introduction

Heterotetrameric sarcosine oxidase (TSOX) is a bacterial enzyme that catalyzes the oxidation of sarcosine (*N*-methylglycine) by molecular oxygen to give an *N*-methylene imine carboxylic acid intermediate and hydrogen peroxide.[1] TSOX has a binding site for tetrahydrofolate (H_4folate).[2,3] In the presence of H_4folate, the rate of sarcosine oxidation is moderately enhanced and the methylene group of the imine intermediate becomes incorporated into H_4folate, yielding 5,10-methyltetrahydrofolate (5,10-CH_2-H_4folate) plus glycine as reaction products. In the absence of H_4folate, the imine intermediate is hydrolyzed by water to yield formaldehyde and glycine (Figure 1).

TSOX, isolated from various bacteria, has a molecular mass of ~180 kDa and is induced to high levels (up to 2% mass) during growth on sarcosine. Its four

Abbreviations used: TSOX, heterotetrameric sarcosine oxidase; H_4folate, tetrahydrofolate; GR, glutathione reductase; RMSD, root mean square deviation; PDB, Protein Data Bank; ICP-MS, inductively coupled plasma mass spectrometry; MSOX, monomeric sarcosine oxidase; EPR, electron paramagnetic resonance; DMGO, dimethylglycine oxidase.

E-mail addresses of the corresponding authors: marilyn.jorns@drexelmed.edu; mathews@biochem.wustl.edu

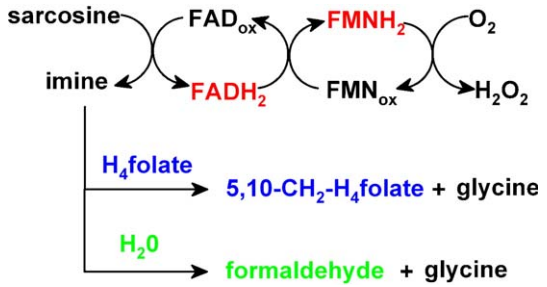

Figure 1. Reactions catalyzed by heterotetrameric sarcosine oxidase.

subunits have molecular masses α = 103 kDa, β = 44 kDa, γ = 21 kDa and δ = 11 kDa. The enzyme contains two non-equivalent flavins per protein molecule, one FAD attached non-covalently and one FMN attached covalently, the latter bound to the β-subunit.[4–6] The enzyme also contains one molecule of NAD. The catalytic function of NAD is unknown.[7] Analytical, kinetic and spectroscopic evidence shows that FAD is the site of sarcosine oxidation and FMN the site of oxygen reduction. Sarcosine oxidation is rate-limiting. Reductive half-reaction studies show that the two electrons transferred from sarcosine are in rapid equilibrium between the two flavins with a biradical being the major component.[8,9] The biradical consists of one neutral (blue) and one anionic (red) flavin semiquinone, as judged on the basis of its visible absorption spectrum. The line width of its electron paramagnetic resonance (EPR) signal indicates a separation of at least 10 Å for the two flavin rings.

Analysis of the amino acid sequence of TSOX indicates the presence of a characteristic ADP-binding motif in the N-terminal portions of both the α and the β-subunits.[10] The β-subunit also shows strong sequence similarity to monomeric sarcosine oxidase (MSOX), which binds FAD covalently, suggesting that this subunit contains the FAD-binding site and that the NAD is bound in the α-subunit. This was corroborated by the successful expression of an individual α-subunit containing NAD.[11] The C-terminal portion of the α-subunit was postulated to bind H_4folate, since it exhibits homology with the T-protein component of the multienzyme glycine cleavage system that also reacts with H_4folate.[10]

An X-ray diffraction analysis of the structure of TSOX was undertaken because of the complexity and multifunctional nature of the molecule. This includes having separate sites for the oxidation of sarcosine (FAD), for the reduction of dioxygen and formation of hydrogen peroxide (FMN) and for the addition of the product methylene group to H_4folate. The aim has been to (1) determine the nature of the intervening medium between the two flavins, their relative orientations and propensity for electron transfer, (2) investigate the putative channeling pathway for transfer of the labile imine product from FAD to the folate-binding site, (3) investigate the role played by NAD+ in the function of TSOX, e.g. whether structural or catalytic and (4) identify the roles played by the γ and δ-subunits of TSOX. We report here the three-dimensional structure of TSOX from *Pseudomonas maltophilia* determined at 1.85 Å resolution. A preliminary account of these findings has been presented.[12]

Results

Structure analysis

Overall structure of TSOX

TSOX is an ellipsoidal molecule of approximate dimensions 120 Å × 85 Å × 65 Å. It consists of the four subunits, α, β, γ and δ (Figure 2).

The α-subunit

The α-subunit is divided into two large separate domains, αA (residues α1–α566) and αB (residues α574–α965); these are connected by a short linker (residues α567–α573) (Figure 3(a)). The N-terminal domain (TSOX-αA), in turn, comprises three sub-domains, αA1 (residues α1–α122), αA2 (residues α123–α444) and αA3 (residues α466–α566) (Figure 3(a)). The latter two sub-domains are connected by a 21-residue coil. The largest sub-domain (αA2) contains the binding site for NAD.

The TSOX αB-domain also consists of three sub-domains, αB1 (residues α574–α632 and α725–α825), αB2 (residues α633–α724 and α826–α869) and αA3 (residues α870–α965). They are arranged in a cloverleaf-like fashion around a central funnel-shaped opening, with three crossover connections between sub-domains αB1 and αB2 (Figure 3(b)).

The αA2 subdomain of TSOX contains a nucleotide-binding motif that consists of a five-stranded parallel β sheet flanked by a pair of α-helices on one side and by a three-stranded antiparallel "β-meander" on the other, characteristic of the glutathione reductase (GR) family of flavoenzymes.[13] Structural alignment of this sub-domain with GR using LSQMAN[14] results in a match of 227 C^{α} positions that show an RMSD of 1.77 Å (Table 1). The large extent of this structural homology, covering nearly the whole of the sub-domain, places it in the subclass GR_1 that includes a number of disulfide reductases[13] similar to GR. In the superimposed molecules, the ADP portion of the NAD cofactor of TSOX is nearly congruent with that of GR, but the nicotinamide and flavin rings of the two proteins are displaced from each other and interact with slightly different portions of the aligned proteins.

The β-subunit

The β-subunit of TSOX contains both covalently bound FMN and non-covalently bound FAD (Figure 4(a)). Like the αA2 sub-domain, the β-subunit contains an FAD-binding motif (a five-stranded